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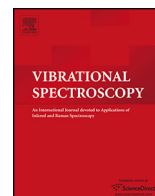
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Preventing damage of germanium optical material in attenuated total reflection-Fourier transform infrared (ATR-FTIR) studies of living cells

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ABSTRACT

Germanium is an important element for ATR-FTIR spectroscopy. It has often been selected as a substrate for live cells FTIR spectroscopy because of its low toxicity for most human cells. However, there is a lack of study on the physical interactions between germanium and the living cells. In this study, we have shown that germanium surfaces are easily damaged when living cells are grown directly in contact for more than 20 h on the element surface. We then proposed a methodology to reduce the damage by coating the surface of the element with a thin layer of biological materials and tested for their capacities to protect polished germanium surfaces from living cell cultures. Fibronectin, collagen and gelatine coated and non-coated, newly polished Ge plates were subjected to 48 h culture of HeLa cells for six times and the degree of erosion by the cell was monitored using optical and atomic force microscopy (AFM). The cells showed normal attachment and morphology to either the treated or untreated surfaces and the viability of the cells were confirmed by trypan blue assay. Furthermore we have tested with an ATR FTIR measurement with a multi-bounce Ge element and found that the coatings were thin enough not to interfere with the measurements. However the degree of erosion (measured by the roughness of the Ge plate after cell culturing) was found to depend on the types of coating used where gelatine coating showed the best protection.

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1. Introduction

Fourier transform infrared (FTIR) spectroscopy is a powerful label-free analytical technique. It is an ideal method for analysing biological materials [1]. Infrared radiation is a low energy wave so that the measurement can be non-destructive and without any influence on the sample. Several studies of FTIR imaging of fixed biological material have shown significant differences between different cell types [2], cancer cells and normal cells [2,3], as well as cancer cells response to chemotherapy drugs [4–6]. However, using living cells not only avoids artefacts due to the fixation process, but also enables to study the living material through time. To measure living cells, the issue about the strong absorbance of water in the mid-IR region has to be overcome. For this reason, transmission cells with very small path lengths have been designed [7–9] and synchrotron radiation is often employed for the measurement of living cells [10–15]. Attenuated total reflection (ATR) was also found to be a suitable measurement mode for this purpose [16,17]. In ATR mode measurements, samples are probed

by the evanescent wave which is generated on the measuring surface when the infrared light undergoes an internal reflection (or multiple internal reflections for a multi-bounce ATR) through the high refractive index ATR element [18]. The ATR FTIR measurement of live cells was achieved by seeding and culturing cells directly on the measuring surface of the ATR element such that the cells are measured in a similar geometrical environment as in a typical cell culturing flask [19]. The path length are small and easily controlled and the sampling depth, which is $\sim 2\text{--}3$ times the depth of penetration, is approximately $0.5\text{--}1.5\text{ }\mu\text{m}$ which probes deep into the attached living cell but without significant interference from the bulk medium above the cells.

The development in the ATR FTIR measurement of live cells allows their study under treatment of drugs *in situ* for time-resolved FTIR spectra of the living cells to be recorded [20]. One of the promising applications of ATR FTIR study is the imaging of living cells in micro ATR mode [21]. Imaging in micro ATR mode has the added advantage of the increased numerical aperture where the light is further focused when approaches the sample through the high refractive index ATR element. A four times improvement in spatial resolution has been demonstrated when using Ge, which has a refractive index of 4, as the ATR element of the objective [22].

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Using this approach, it has been shown that major cell organelles such as nucleus and endoplasmic reticulum can be resolved [22]. Ge is an important ATR element and it is universally used for micro ATR imaging. The surface properties of Ge are different to the standard cell culturing flasks which living cells may interact differently. A previous study has shown that cells can be cultured on Ge surface with no observable difference in morphology when compared to cell grown in tissue culture flask [23] and the attachment of cells was improved when the ATR surface was coated with a thin layer of collagen [21]. However, the coating of Ge appears to be more important than simply improving the attachment of cells. In this work, we report on the possible damage to the bare Ge ATR element by the attached living cells. We have assessed the degree of damage by visible microscopy as well as atomic force microscopy after a single and repeated cell culturing on the optical element. This is important because the degree of damage is thought to be directly related to the scattering of light and loss of throughput. We have also measured the protective effect of various extracellular matrix components routinely used in tissue culture such as gelatin, collagen and fibronectin [24,25] as coating on Ge in order to propose a solution to reduce the degree of damage to the Ge surface.

2. Materials and methods

2.1. Optical materials and coatings

Newly polished germanium windows of 1 mm thick and 25 mm of diameter (surface area $\sim 491 \text{ mm}^2$) were used as obtained from Crystan Ltd (UK). The coatings applied to the Ge windows were

gelatine type 2 tissue culture grade (2% solution from Sigma, UK), collagen from calf skin type I for tissue culture (Sigma) and fibronectin from bovine plasma (Calbiochem, UK). Gelatine coating was applied at 2 mg/cm^2 , or $490 \mu\text{L}$ of 2% solution per window. Fibronectin was applied at a $5 \mu\text{g/cm}^2$, by applying $245.4 \mu\text{L}$ of a 0.1 mg/mL solution per window. Collagen was dissolved as recommended by the supplier to a working solution of 0.01% , which $490 \mu\text{L}$ were applied to obtain a coating of $10 \mu\text{g/cm}^2$. After application of the coating solutions the windows were left to dry for 2 h at 35°C .

For FTIR measurements of live cells, a temperature controlled multi-(i.e. 20) bounce ATR accessory trough plate (HATR, Pike technologies) and germanium ATR element ($80 \text{ mm} \times 10 \text{ mm} \times 2 \text{ mm}$, Pike technologies) with an angle of incident of 45° were used. The path length in the living cells produced from this accessory in the $2000\text{--}1000 \text{ cm}^{-1}$ region is approximately $3\text{--}6 \mu\text{m}$ with a depth of penetration of $\sim 0.32\text{--}0.64 \mu\text{m}$. The trough plate has a measurement area of $\sim 500 \text{ mm}^2$ where the live cells can attach and be measured. The coatings of the ATR plate were applied similarly to the optical windows but volumes adjusted to the growth area of 500 mm^2 .

2.2. Live cells preparation

HeLa (ATCC#CCL-2) and PC3 (ATCC#CRL-1435) were maintained in T25 cell culture flasks using RPMI medium supplemented with 10% FBS and 2 mM L-glutamine, in a 5% CO_2 environment at 37°C . The cells were trypsinised and harvested when they reached $\sim 80\%$ confluence. The cells were then centrifuged into a pellet and re-suspended in L15 medium (Sigma Aldrich) supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, 100 U/mL

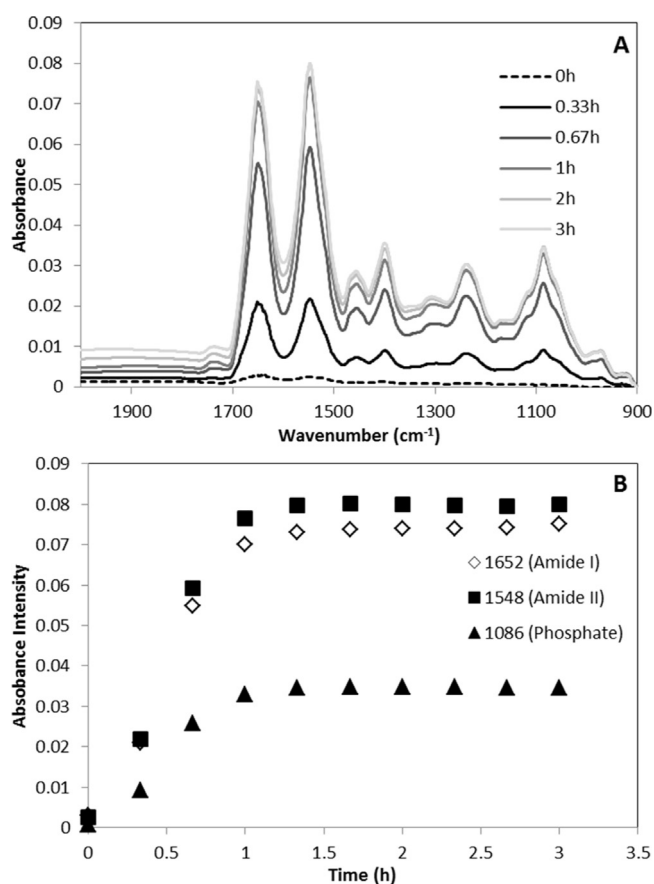


Fig. 1. FT-IR spectra of cells in the first three hours after seeding in the ATR element. (A) absorbance spectra and (B) absorbance intensity of the main peaks in function of time. As background the spectrum of medium was used.

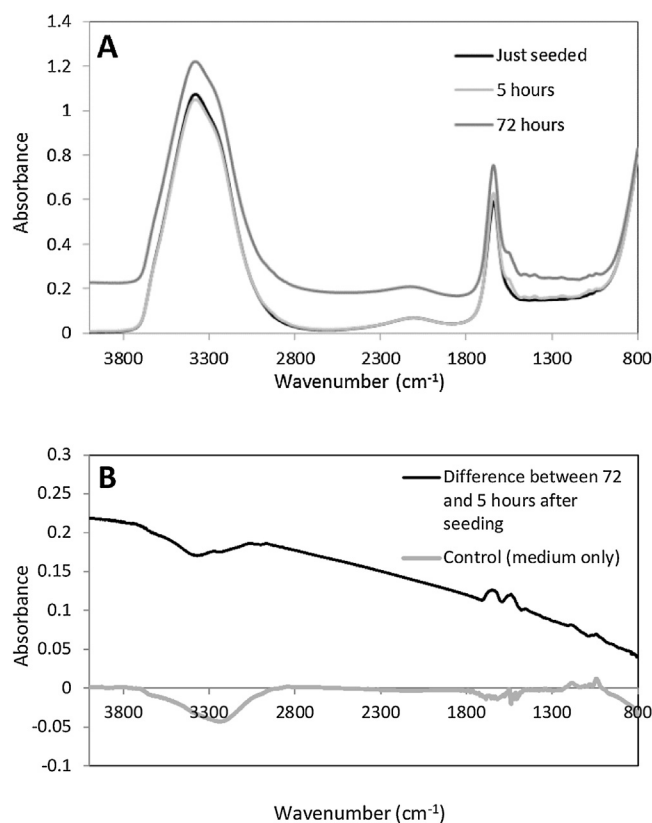


Fig. 2. Baseline shift observed in (A) ATR-FTIR spectra of cells just seeded, 5 h and 72 h after seeding, using as background a spectrum obtained with the empty crystal before seeding, and (B) difference spectrum between 5 h and 72 h of the cell attachment (dark line) and control (medium only, grey line).

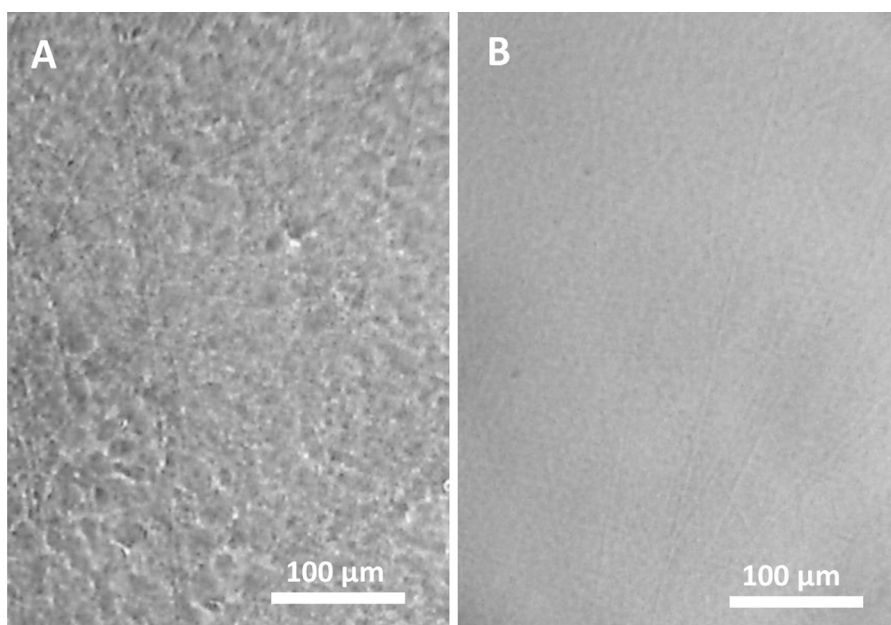


Fig. 3. Reflective visible images of a germanium ATR crystal surface after cell culture, showing a roughness where the cells were attached to the crystal (A) and a smooth surface in the area not exposed in the cell culture (B).

streptomycin. L15 was used during the ATR measurement to allow closed chamber culturing with air as the gaseous phase, and therefore it was also used when cells were seeded on germanium windows. The cell suspension was diluted in medium to reach a cell density of $\sim 2.4 \times 10^6$ cell per mL.

2.2.1. Cell culturing on germanium windows

Germanium windows were placed in 6 well plates and 0.5 mL of the resulting cell suspension were seeded on the windows. The

cells were allowed to settle for one hour, after which 2 mL of medium were added to each well and the cells were left to grow at 35 °C. The confluence was verified using reflective optical microscope with a 10× objective (NA 0.25) coupled with a camera. The cell culture was maintained till 48 h after seeding; the cells were then stained with trypan blue to test the viability, and then removed by washing the windows with distilled water using a micropipette. The surface of the washed windows was imaged by

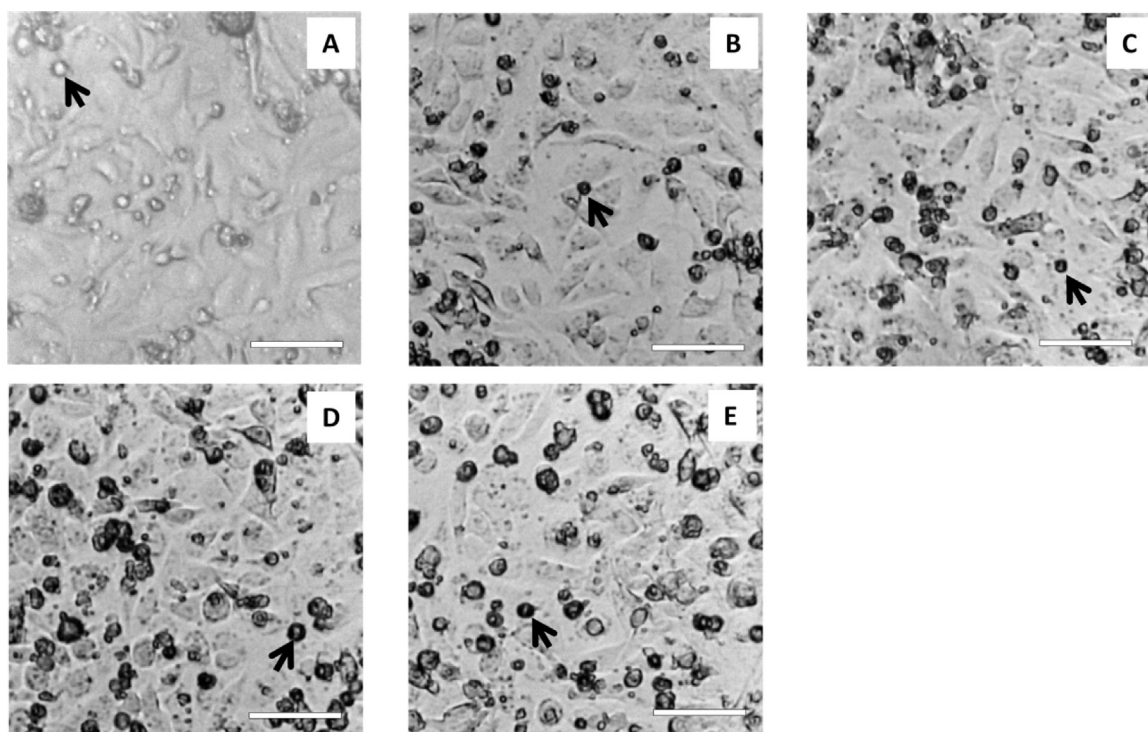


Fig. 4. Reflective visible images of HeLa cells growing on plastic (A), uncoated germanium (B) and on germanium coated with gelatine (C), collagen (D) and fibronectin (E) for 48 h. Dark and round cells stained with trypan blue as shown by arrows for each figure, as the backgrounds in A (plastic) and B–E (germanium) provide different contrast. Scale bar 100 μm.

optical microscopy using a reflective microscope and by atomic force microscopy (AFM).

2.2.2. ATR measurements of cell growth and doxorubicin treatment

Detailed procedures can be found in the previous work [19]. In brief, two millilitres of culture medium were applied directly onto the multi-bounce, temperature controlled ATR (at 35 °C) and a spectrum was measured. Five hundred microlitre of cell suspension (i.e. ~1.2 million of cells) were seeded directly onto the ATR trough which was then sealed and maintained at 35 °C, covered with a lid (also temperature controlled at 35 °C) to prevent evaporation. After incubating overnight, the cell seeding density ensured continual growth without reaching confluence (<90%) of cells adhered to the measuring surface. The attachment of cells and confluence were verified using reflective optical microscope. For assays on the response of cells to doxorubicin, 1 μ M doxorubicin was applied to the HeLa cells cultures after the overnight growth on the ATR surface, similarly to the previous reported²⁶ but instead of using a ZnS ATR element as in previous work, a Ge ATR was used.

2.3. FTIR measurement of samples

Continuous scan FTIR spectrometer (Frontier, Perkin Elmer Ltd, UK) fitted with a room temperature deuterated triglycine sulfate (DTGS) detector was used to acquire the IR spectra with a scanning

time of 11 min, a spectrum was measured every 20 min. All measurements were acquired with a spectral resolution of 8 cm^{-1} and a spectral range of 4000–900 cm^{-1} and a mirror speed of 0.2 cm/s. The strong Norton-Beer apodization function and self-phase correction was chosen for the process of the interferogram. Spectra were analysed without further data retreatment except those for principal component analysis where spectra were baseline corrected based on wavenumber 1990, 1797 and 940 cm^{-1} using the Spectrum 10 software (Perkin Elmer). Full spectra of the cells were obtained, using as background the spectrum of culture medium, on the coated or uncoated Ge as stated. Water vapour peaks were subtracted from each spectrum using previously obtained spectra of water vapour, by capturing a sample-free spectrum in ambient conditions against a background with the environment dried with desiccant.

2.4. AFM measurements

AFM was carried out in tapping-mode using Bruker (ex-Veeco) Dimension ICON high-end SPM, Nanoscope V controller (Veeco, Cambridge, UK) with a Silicon AFM tip of resonant frequency of 160 kHz and a force constant of 5.5 N/m, to image an area of 93 $\mu\text{m} \times 93 \mu\text{m}$ with a resolution of 512 \times 512 pixels and a scan rate of 0.7 Hz. All AFM images were performed in air.

Surface roughness parameter root mean square (Rq) was calculated, as well as the 95% maximum depth. (Bruker's Nanoscope V and Microsoft[®] Excel 2010) The 95% maximum depth was obtained by excluding the 2.5% lowest and highest measurements as possible outliers. All measurements were taken in triplicate and results are presented as average \pm standard deviation.

2.5. Statistical analysis

Microsoft[®] Excel 2010 was used and the results were expressed as means \pm standard deviation. Additional analysis of variance (ANOVA) was performed with $P=0.05$. PCA was carried out using in-house software developed for analysis of spectra for a previous study [26], using the python programming language with numpy and scipy for calculations, and matplotlib for visualization. Spectra were truncated to 2000 cm^{-1} and 900 cm^{-1} wavenumber followed by baseline correction before the PCA.

3. Results and discussion

3.1. Multi-bounce ATR measurements

FTIR measurement results of the cells attaching and growing on the multi-bounce Ge ATR element are shown in Fig. 1 where culture medium was used as the background spectrum to highlight the attachment and growth of the cell on the Ge surface. During the first few hours after seeding the cells on the bare Ge ATR element, the absorbance associated with cellular materials was increasing (Fig. 1A) demonstrating that the cells were attaching and spreading on the ATR surface, forming and achieving a good contact. The spectral data obtained showed similarities to a previous study where breast cancer cells were grown on an ATR surface [27]. A separate study has shown that due to the small probing depth of ATR measurements, there will be no significant absorbance from the cells if they are not attaching to the ATR surface even if they sediment to the surface of the ATR element [26]. The absorbance of the cells reached a plateau after 60 mins (Fig. 1B) marking the end of the cell attachment process. After the attachment of the cells, there is a continual but small increase in the absorbance of the cell spectrum from cell growth (Fig. 1B), as observed in a previous study [19]. All these observations are similar to the cells grown in tissue culture flasks and previous results from multi-bounce ATR

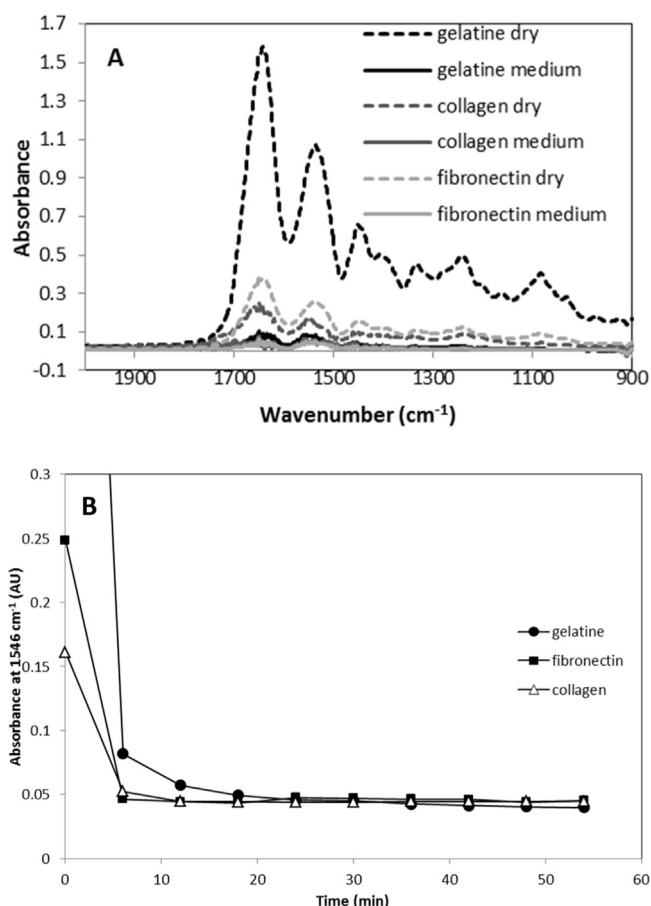


Fig. 5. FT-IR spectra of gelatine, collagen and fibronectin coatings on the ATR element before adding medium (dry) and after adding medium (medium). "Dry" spectra were obtained using as background the spectrum of the empty ATR, while the background for "medium" was a spectrum of medium without coating. (A) shows the spectra of "dry" coatings and after the addition of medium, and (B) shows the height of the band at 1546 cm^{-1} (Amide II) in the first 55 min after the addition of medium.

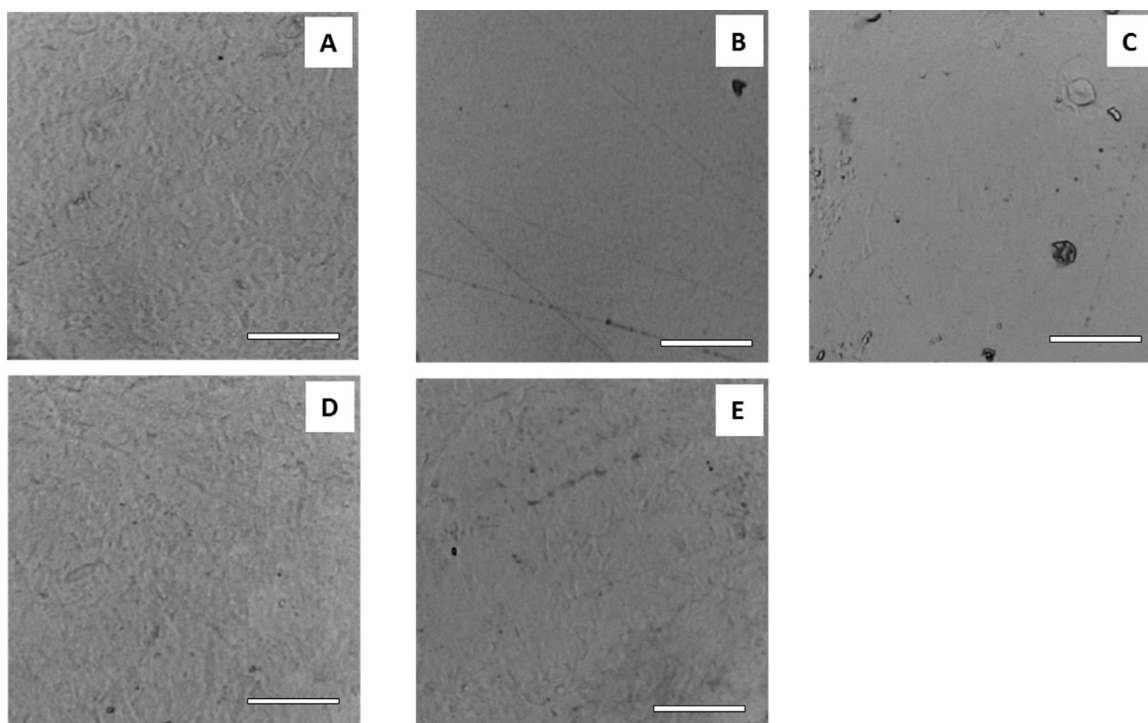


Fig. 6. Reflective visible images of germanium windows after undergoing cell culture for 6 times, (A) no coating (control), (B) uncoated submitted to medium without cells, coated with (C) gelatine, (D) fibronectin, (E) collagen. Scale bar 100 μm .

study of HeLa cells using a ZnS ATR element [26]. Fig. 2A shows the FTIR spectra of the cells with a clean Ge element as the background. The spectrum of the seeded cell immediately after seeding shows mostly the spectrum of water in the medium because cells were not yet attached. The spectrum of living cells in medium can be clearly seen 5 h after seeding which has a baseline that is similar to the spectrum measured immediately after seeding. However, approximately 20 h after seeding the living cells, a significant increase of a non-specific absorbance, in the form of a baseline shift, can be observed which continued throughout the experiment. After 3 days of cell culturing, the non-specific absorbance or baseline lift reached over 0.2 at 4000 cm^{-1} (Fig. 2). The baseline shift, highlighted by the difference spectra between the cells seeded for 5 h and 72 h is shown in Fig. 2B and is compared to a control measurement when medium without cells was incubated. The difference spectrum shows amide I and amide II bands on a strong baseline which resembles a typical feature of a non-specific scattering where a greater loss of IR intensity is found on the shorter wavelength than the longer wavelength. The difference spectrum showed a small decrease in the OH stretching mode absorbance because as the cells continue to grow, as shown by the increase in amide II band, the amount of water within the sampling volume decreases. The image of the surface of the Ge ATR element captured after removal of the cells by trypsin (Fig. 3) shows large degree of damage by the growing cells. The mechanism of the attack of Ge by living cells could be an interesting topic of biochemical study but it is beyond the scope of this work. The damage by the cells created a roughened surface on the Ge ATR element which increased the scattering loss of IR radiation. A reduction of the overall throughput of IR light through the Ge ATR element by $\sim 40\%$ can be observed even when the cells were removed and the Ge surface has been cleaned, showing that the permanent loss of throughput is related to the damage of the ATR surface by the cells. This is also supported by the fact that on other materials that are not damaged by cell culturing the decrease of throughput is not observed, as can be seen on the spectral data of

previous studies from our group using HeLa cells on a ZnS ATR element [26]. The lack of scattering loss in the first 20 h of incubation indicates that the damage of Ge by living cells happens after the cells have been attached for over ~ 20 h which explained why this damage was not observed in previous studies [21] where a much shorter duration of cell incubation was used. However, many drug-cell interaction studies expose cells to drug in a period of 48 h or longer. A method to reduce cellular damage to the optical material is needed for the future application of micro ATR imaging study of drug-cell interactions.

3.2. Protective coatings

The method proposed here to protect the Ge surface from damage is through coating the germanium with materials that would also enable cell attachment and cell measurement by ATR-FTIR. Three materials used commonly in tissue culture were chosen and HeLa cells used as an example which is shown to be able to attach and grow on Ge coated with gelatine, collagen or fibronectin, as shown in Fig. 4 where the images were captured 48 h after seeding.

ATR-FTIR spectra of the coatings were collected after the drying step, before and after adding culture medium. As it can be seen in Fig. 5, the spectrum of each of the three coatings was higher when the coatings were dry than when the medium was added, which is due to the dissolution of the coating material (water-soluble protein) in the medium. However, the absorbance remained at 0.05 after 24 min which is higher than what is expected should the protein dissolve completely (if the protein dissolved completely the concentrations should be $490\text{ }\mu\text{g/mL}$ for gelatine, $12.4\text{ }\mu\text{g/mL}$ for fibronectin and $2.45\text{ }\mu\text{g/mL}$ for collagen, all of which resulted in an absorbance of lower than 0.005 using the same accessory), which means that a thin layer of adsorbed protein remained on the Ge element. The spectra of the three coatings 24 min after the medium was added reached very similar amide II absorbance values, suggesting that the coatings have similar final thickness on

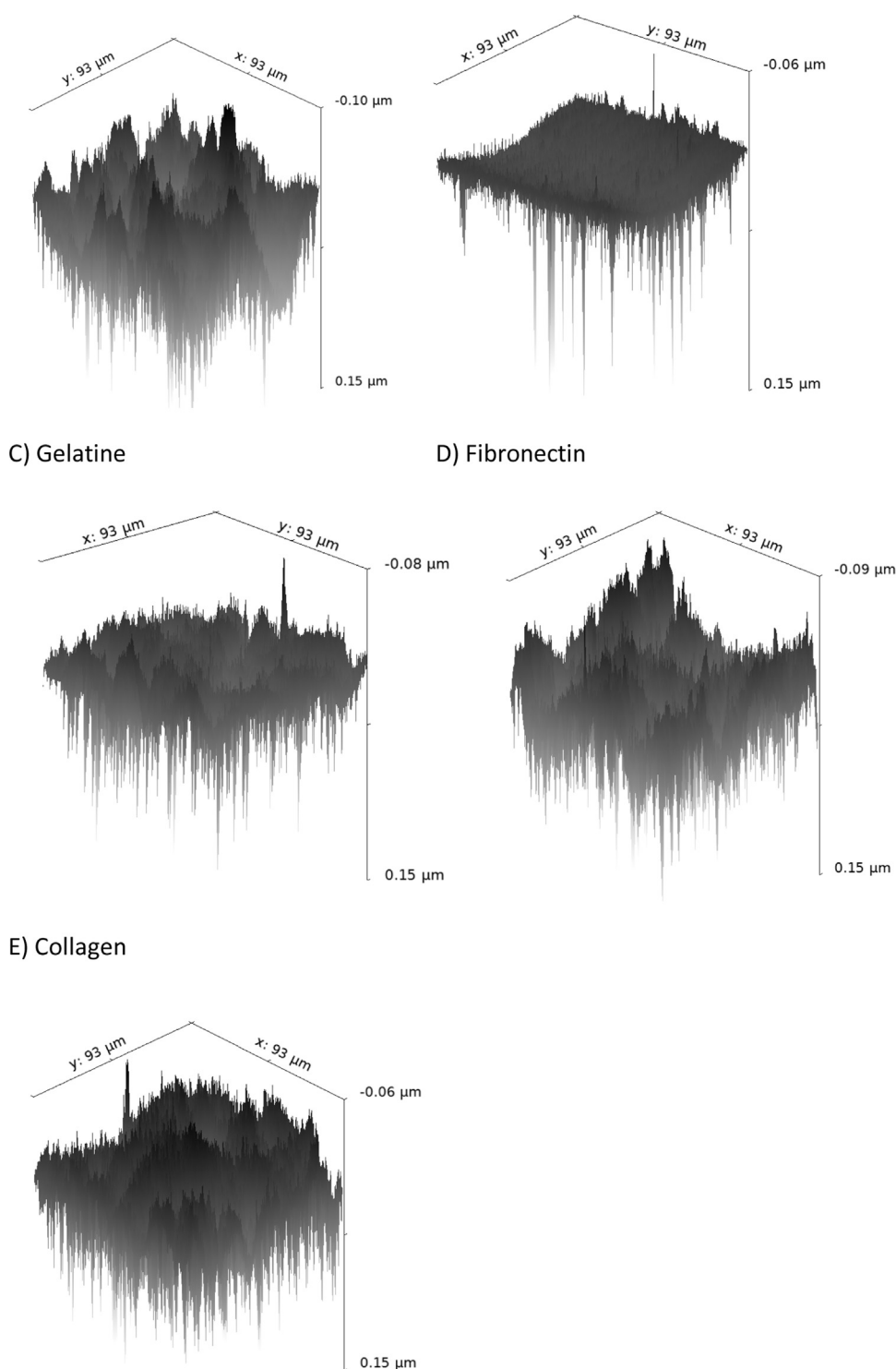


Fig. 7. AFM mapping of germanium windows after HeLa cells were seeded 6 times. A no coating; B, gelatine; C, fibronectin; D, collagen; E, culture medium without cells.

the Ge element in the medium (Fig. 5B), in spite of having been applied at different concentrations.

From the morphology of the cell it is clear that HeLa cells proliferated on the Ge surface to high confluence showing no signs of toxicity by the bare germanium or by germanium with any of the coatings. Trypan blue was added to the cell culture and as can be seen in Fig. 4 where the cells attached to the surfaces, most cells are not stained indicating that they are not undergoing apoptosis. Note

that the main difference observed in the images between Fig. 4A and B–E are due to the different background. Fig. 4A was measured against the transparent background of the tissue culture flask whereas Fig. 4B–E were measured against the reflective background of Ge which resulted in the higher contrast. The stained cells over the Ge discs show very dark round structures with a small light spot in the centre while on the tissue culture flask, they were shown with a much lighter spot in the centre. Other darker

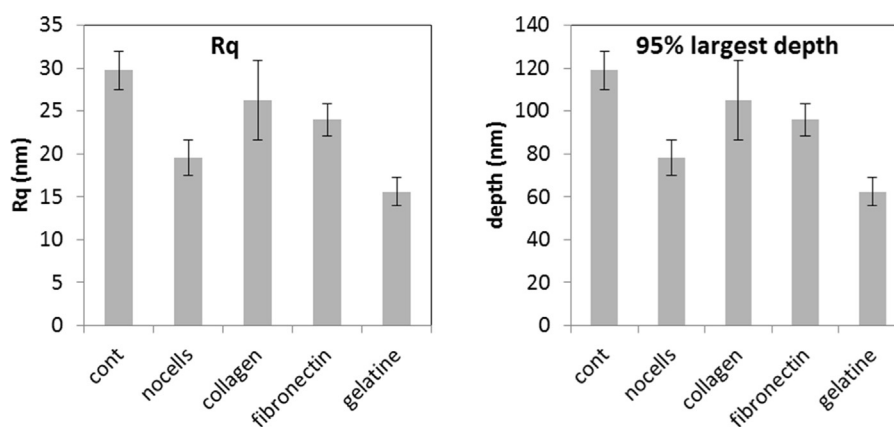


Fig. 8. Surface roughness parameters root mean square (Rq) and 95% maximum depth of grooves after cells being seeded 6 times in germanium plates. Error bars represent the standard deviations of three independent measurements.

cells on top may be due to the ageing of the culture, as it was confluent for approximately 24 h.

3.3. Roughness of Ge surfaces

Cells were cultured 6 times in newly polished uncoated and coated Ge windows (as in Fig. 4), and after each time the cells were removed with trypsin and the surfaces were recoated for re-seeding. This is to show if the damage on Ge can be prevented for multiple usage. Exposing the newly polished Ge window in trypsin for 5 min did not show any observable effect on the surface. After the 6th culture, the surfaces were washed and observed in the optical microscope. As shown in Fig. 6, marks were visible where the cells were anchored, especially in the bare window (Fig. 6A). These marks have been noticed before (Ge crystal used for several ATR cell experiments shown in Fig. 3) and increase light scattering, and consequently the noise in the spectrum. Similar marks can be observed on the Ge coated with fibronectin and collagen but appeared to be less extensive in the latter. Ge coated with gelatine (Fig. 6C) showed the smoothest surface which has a similar appearance to the control Fig. 6B. To quantify the roughness of the germanium plates, the plates were analysed by AFM after the 6th culture (Fig. 7). It can be seen from the image that the bare surface showed the largest variations in height across the imaged area while the coated surface showed smaller variation in height after cell culturing. The size of these defects from peak to trough as shown in the AFM images is on the order of 15 μm . The roughness was quantitatively compared by the parameters Rq (root mean square of values) and the 95% highest depth (Fig. 8). The 95% highest depth consists in the difference between highest and lowest values, discounting the 2.5% highest and 2.5% lowest values to account for possible outliers due to noises. The values observed approach a normal distribution. All calculations were based on non-smoothed AFM data because the roughness was found to be dominated by the micron-sized features. Smoothing of the AFM maps, which removes the nano-sized roughness, did not result in significant difference in the calculated values. After being subjected to cell culture for 6 times, both Rq and 95% largest depth are lower for the coated Ge plates compared to the control plate. Fig. 8 showed that the Rq and 95% largest depth values for the gelatine coated Ge are similar to the plate treated with no cells.

The result has shown that, while fibronectin and collagen may protect to a certain extent against the living cells, gelatine offers the best protection with the roughness measurement values similar to the plate without cells grown on top. This is in agreement with the visible images shown in Fig. 7. This suggests that the

gelatine coating could protect the surface of the germanium crystal for multiple exposures to the culture conditions. In fact, scratches in the uncoated germanium surface, not submitted to cell culturing, were observed in the optical microscope and can be seen in Fig. 6B, while in the gelatine coated plate these scratches seem less evident (Fig. 6C). The depth of the marks on the surface measured by AFM also confirms that the depth of the deepest scratches in the uncoated window not subjected to cells was 17 nm higher than the deepest marks on the gelatine coated surface (Fig. 8).

3.4. ATR measurements of cells growing on coated surfaces and treated with doxorubicin

As the purpose of coating the germanium is to protect ATR crystals during FT-IR measurements, the coatings were tested to confirm that not only the cells could attach to the coated Ge surface, but also the spectra of the live cells could be obtained without strong interferences. As shown in Fig. 9A and B, the spectra of the cells are similar in the presence of any of the coatings under evaluation and with the uncoated germanium ATR crystal (control) showed <10% stronger absorbance of the cell and an elevated baseline. The results show that the coating is thin enough to allow majority of the signal from the living cells to be measured and offers protection to the Ge surface.

HeLa cells grown on a gelatine-coated ATR surface were treated with 1 μM doxorubicin and subsequently analysed for 21 h, acquiring 1 spectrum/h. The concentration of 1 μM was chosen as for its clinical significance, as it is the therapeutic concentration of doxorubicin in the bloodstream [28]. As can be observed in Fig. 9C, the difference spectra from the moment the drug was added resemble the spectrum of the whole cells, suggesting that the cells are detaching from the ATR crystal as all the absorbance due to their components are decreasing in the same proportion. These differences were confirmed by analysing the spectral data by PCA (Fig. 9D and E). PC1 shows that the overall absorbance of the cell decreased rapidly in the first 7 measurements (7 h) followed by smaller changes, represented by PC2, in the next 17 h. The results are in agreement with the previously reported for doxorubicin on uncoated ZnS surfaces [26], demonstrating that the coating did not affect the reaction of the cells to doxorubicin and the ability of ATR FTIR to observe the cellular changes *in situ*. The coating of germanium ATR elements would therefore allow microspectroscopy studies of live cells with improved spatial resolution. In recent years FTIR imaging studies have shown the potential of this technique in obtaining

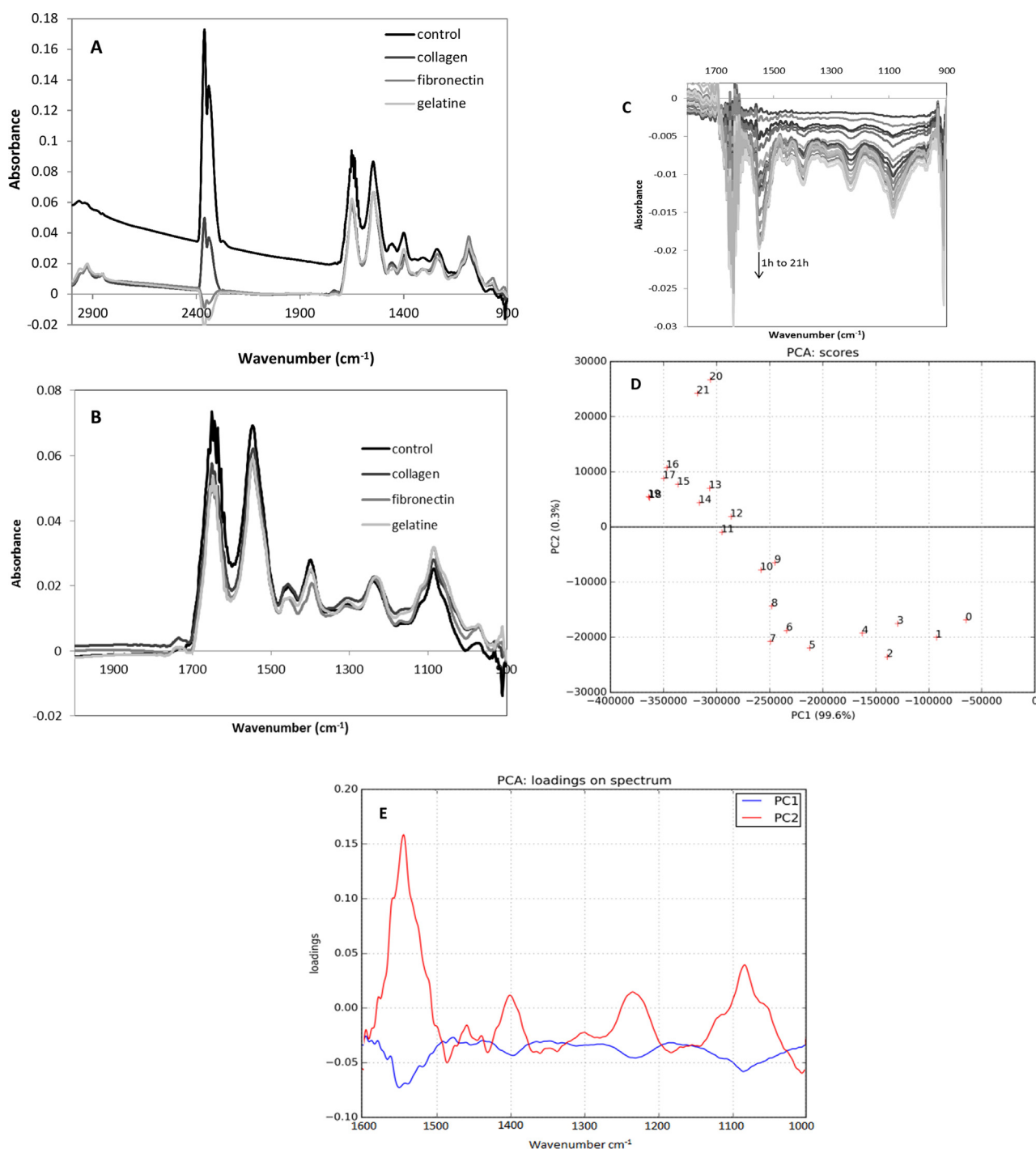


Fig. 9. FTIR analysis of HeLa cells in coated plates. Spectra of HeLa cells on coated and uncoated (control) ATR elements 17 h after seeding (overnight incubation), as background the spectrum of medium on the coated ATR with the respective coating was used (A) before baseline correct and (B) after baseline correction and truncated to 2000 cm⁻¹ to 900 cm⁻¹ region, (C) difference spectra after applying doxorubicin (1 μM) to HeLa cells grown in a gelatine-coated Ge ATR surface, using as background the first spectrum immediately after the application of the drug, (D and E) PCA scores and loadings for the spectra shown in (C) where the number on the score plot represent the number of hour of exposure.

biochemical profiles of cells for biomedical sciences. Significant changes were found in cells in fixed tissue related with the response of melanoma cells to a chemotherapy drug [29], distinguishing cancer cells in lymph node metastases [3], distinguishing different types of healthy cells from breast tissue as well as differentiating cancer and healthy cells [2]. The possibility of imaging living cells cultured directly on a Ge ATR

element will allow *in situ* high-resolution measurements through a time course, resolving where and when the changes occur, and avoid possible artefacts due to the fixation and drying process. The present study suggests that the use of gelatine coatings can decrease the damage of Ge surfaces by cell cultures, enabling the analysis of cells growing on Ge surfaces for several days, and the repeated use of the Ge crystal for that purpose, as the cost of the

optical materials, especially for micro-ATR measurements, is generally an important factor in spectroscopic studies.

4. Conclusion

Germanium surfaces coated with tissue culture coating material prevents damage by culturing live cells in live cells ATR-FTIR studies. Gelatine, fibronectin or collagen coated Ge surfaces of optical elements enable cells to anchor and grow, and to take FT-IR measurements of the cells without noticeable interference. Gelatine coating showed the most efficient protection after repeated use, preventing not only the damage by the live cells but also the appearance of scratches in Ge surfaces.

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